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TRANSLATION NO. 2573

DATE: Oct 29, 1969

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PHENOTYPIC MANIFESTATIONS OF CERTAIN GENETIC PROPERTIES OF HERPES SIMPLEX VIRUS DEPENDING ON CONDITIONS OF CULTIVATION

[Following is the translation of an article by A. B. Germanov and M. I. Sokolov, Institute of Virology imeni D. I. Ivanovskiy, AMN USSR, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology) 13: 611-18, 1968. It was submitted on 8 Feb 1968.]

A study was made of the influence of incubation temperature and type of cell culture on the plaque-forming capacity and resistance to sulphated polysaccharides on the part of various mutants of the $L2_{K5}$ strain of the herpes virus. Mutants which were thermoresistant and resistant to dextran sulphate , just as the initial strain, on a culture of chick embryo fibroblasts at 28° were more sensitive to sulphated polysaccharides than at 37°. Sensitivity to these inhibitors on a culture of human embryo fibroblasts was manifested to a greater degree than on a culture of chick embryo fibroblasts. Mutants, unable to form plaques on the latter of these 2 cultures in the absence of DEAE-dextran, were no different phenotypically in plaque-formation than the initial strain , and on a culture of human embryo fibroblasts formed plaques both with the presence of DEAE-dextran in the covering and without it. Mutants, forming "blotched" plaques on a culture of human embryo fibroblasts, were no different from the initial strain on a culture of chick fibroblasts, causing the formation of hyaline plaques.

At the present time it is well known that the nature of manifestation of a gene may vary considerably with a change of external conditions, i.e., one and the same genotype may be manifested differently phenotypically. In particular, on various strains of the herpes virus it has been shown that the temperature of incubation [8], type of cell culture used for reproduction of the virus [11, 12], and composition of the nutrient layer [10, 13, 16] have an influence on the capacity to form plaques. In regard to the sensitivity of the herpes virus to sulphated polysaccharides of agar the data from the literature are contradictory [7, 13, 15]. The influence of various conditions of cultivation on the phenotypic manifestation of sensitivity to sulphated polysaccharides has not been studied sufficiently for the herpes virus.

Taking this into consideration, we studied the change of phenotypic manifestation of the characteristic of sensitivity to inhibitors of agar and dextran sulfate, and also the morphology of plaques in mutants of the herpes simplex virus isolated by us.

Materials and Methods

The work was carried out with the plaque-forming $L2_{K5}$ strain of the herpes simplex virus and its mutants: 1) "plaque-free" (spontaneous - fpp_1 and induced by hydroxylamine - fpp_{10}); 2) resistant to dextran sulfate (spontaneous DS_1^R and DS_3^R); 3) forming "blotched" plaques (spontaneous mt_1 and mt_2); and 4) thermoresistant (spontaneous TR_1 and induced by hydroxylamine TR_{10}). Methods of isolation and certain genetic peculiarities of the mutants used were described by us earlier [3-6].

For titration of the virus we used the method of plaques under an agar covering in the method of Porterfield and Allison [10] with a slight modification, which amounts to the following. With the help of a solution of Gey C the pH was brought to 7.8 and then stabilized with an equal volume of 0.1 M tris-buffer. Chick embryonic extract was substituted for an equal volume of lactalbumin hydrolyzate and neutral red was introduced with the second layer of agar. For infection we used a monolayer culture of chick embryo fibroblasts or human embryo fibroblasts. The cultures were infected by the generally accepted method. For studying the influence of sulphated polysaccharides on plaque-forming capacity into the agar covering we introduced: 1) DEAE-dextran in a concentration of 0.5 mg/ml for removing the inhibiting action of the agar polysaccharides; 2) dextran sulfate (molecular weight $2 \cdot 10^6$) in a concentration of 1-2 mg/ml, inhibiting the formation of plaques by the initial strain. Incubation was carried out at 28 and 37°, the upper layer of agar with neutral red was added on the 7th and 4th days respectively. For investigating the influence of dye on plaque-forming capacity, the infected monolayer was stained also on the 4th, 5th, 6th, and 7th days of incubation at 37°.

Results

In preliminary experiments we showed that the initial strain of $L2_{K5}$ possesses a low sensitivity to sulphated polysaccharides of agar [2] during cultivation on a monolayer of chick embryo fibroblasts at 37°. The addition of DEAE-dextran to the agar covering in a concentration of 0.5 mg/ml does not significantly influence the size of plaques, which on the 5th day of investigation comprised 1-2 mm, but increases the effectiveness of seeding by 30-40%. During incubation of the initial strain on the stated culture at 28° we detected a clearly expressed suppression of the action of the agar inhibitors on the capacity of the virus to form plaques. Under these conditions DEAE-dextran exerted a considerable influence both on the size and on the number of plaques formed (table 1, figure 1, see inset).

Table 1

Influence of temperature of incubation on the sensitivity of strain $L2_{K5}$ and its mutants to sulphated polysaccharides

(a) Штамм	(b) Температура инкубирования (в градусах)	Концентрация (в мг/мл) в покрытии (c): ДЭАЭ-декстрана (d)	Декстрансульфата (e)	(f) Титр на культуре фибробластов куриного эмбриона (в БОЕ/мл)	(g) Эффективность посева (в %)	Размер бляшек (в мм) (h): на 5-е сутки (i)	на 8-е сутки (j)
(k) Л2$_{ис}$	37	0,5	0	$7,3 \cdot 10^8$	100	1—2	—
	37	0	0	$5,6 \cdot 10^8$	76,7	1—2	—
	37	0	1	$1,8 \cdot 10^8$	24,7	<0,5	—
	37	0	2	0	0	Нет (l)	—
	28	0,5	0	$7,1 \cdot 10^8$	97,2	0,5—1	2—3
	28	0	0	$1,1 \cdot 10^8$	15,0	<0,5	0,5—1
	28	0	1	0	0	Нет (l)	Нет (l)
fpp$_1$	37	0,5	0	$1,3 \cdot 10^8$	100	0,5—1,5	—
	37	0	0	0	0	Нет (l)	—
	28	0,5	0	0	0	»	Нет (l)
fpp$_{10}$	37	0,5	0	$6,9 \cdot 10^8$	100	0,5—1,5	—
	37	0	0	0	0	Нет (l)	—
	28	0,5	0	0	0	»	Нет (l)
DS$_1^R$	37	0,5	0	$2,6 \cdot 10^8$	100	1—2	—
	37	0	0	$2,4 \cdot 10^8$	92,3	1—2	—
	37	0	2	$2,2 \cdot 10^8$	84,6	0,5—1	—
	28	0,5	0	$2,5 \cdot 10^8$	96,1	0,5—1	2—3
	28	0	0	$4,5 \cdot 10^8$	17,3	<0,5	0,5—1
	28	0	2	0	0	Нет (l)	Нет (l)
DS$_3^R$	37	0,5	0	$4,5 \cdot 10^8$	100	1—2	—
	37	0	0	$4,5 \cdot 10^8$	100	1—2	—
	37	0	1	$4,4 \cdot 10^8$	97,7	1—2	—
	28	0,5	0	$4,2 \cdot 10^8$	93,3	0,5—1	1,5—2
	28	0	0	$3,1 \cdot 10^8$	18,0	<0,5	0,5—1
	28	0	1	0	0	Нет (l)	Микробляшки (m)
TR$_1$	37	0,5	0	$2,8 \cdot 10^8$	100	1,5—2	—
	37	0	0	$2,1 \cdot 10^8$	75	1,5—2	—
	28	0,5	0	$2,7 \cdot 10^8$	96,4	0,5—1	2—3
	28	0	0	$4,4 \cdot 10^8$	15,7	<0,5	0,5—1
TR$_{10}$	37	0,5	0	$1,6 \cdot 10^8$	100	1—2	—
	37	0	0	$1,1 \cdot 10^8$	68,7	1—2	—
	28	0,5	0	$1,6 \cdot 10^8$	100	0,5—1	2—3
	28	0	0	$2,5 \cdot 10^8$	15,6	<0,5	0,5—1

Designation: - tests not conducted or not considered.
Key: (a) Strain; (b) Temperature of incubation (in degrees); (c) Concentration (in mg/ml) in covering; (d) DEAE-dextran; (e) dextran sulfate; (f) Titer on culture of chick embryo fibroblasts (in PFU/ml); (g) Effectiveness of seeding (in %); (h) Size of plaques (in mm); (i) on 5th day; (j) on 8th day; (k) $L2_{K5}$; (l) None; (m) Microplaques.

Table 2

Influence of cell culture on the sensitivity of strain $L2_{K5}$ and its mutants to sulphated polysaccharides

(a) Штамм	(b) Клеточная культура	Концентрация (в мг/мл) в покрытии (c): (d) ДЭАЭ-декстрана	(e) декстран сульфата	(f) Титр (в БОЕ/мл)	(g) Эффективность посева (в %)	(h) Размер бляшек на 5-е сутки (в мм)
(i) $L2_{K5}$	ФКЭ С	0,5	0	$9,6 \cdot 10^5$	100	1—2
		0	0	$6,4 \cdot 10^5$	66,6	1—2
		0	1	$1,5 \cdot 10^5$	15,6	<0,5
	ФЭЧ Н	0,5	0	$9,9 \cdot 10^5$	100	2,5—4
		0	0	$3,4 \cdot 10^5$	34,3	1—2
		0	1	0	0	Нет (j)
fpp_1	ФКЭ С	0,5	0	$1,8 \cdot 10^6$	100	0,5—1,5
		0	0	0	0	Нет (j)
	ФЭЧ Н	0,5	0	$1,7 \cdot 10^6$	100	2,5—4
		0	0	$5,9 \cdot 10^5$	34,7	1—2
fpp_{10}	ФКЭ С	0,5	0	$8,4 \cdot 10^5$	100	0,5—1,5
		0	0	0	0	Нет (j)
	ФЭЧ Н	0,5	0	$8,7 \cdot 10^5$	100	2,5—4
		0	0	$4,1 \cdot 10^5$	47,1	1—2
DS_1^R	ФКЭ С	0,5	0	$4,1 \cdot 10^6$	100	1—2
		0	0	$3,8 \cdot 10^6$	92,6	1—2
		0	2	$3,5 \cdot 10^6$	85,3	0,5—1
	ФЭЧ Н	0,5	0	$3,8 \cdot 10^6$	100	2,5—4
		0	0	$1,2 \cdot 10^6$	31,5	1—2
		0	2	0	0	Нет (j)
DS_3^R	ФКЭ С	0,5	0	$8,6 \cdot 10^5$	100	1—2
		0	0	$8,4 \cdot 10^5$	97,6	1—2
		0	2	$8,1 \cdot 10^5$	94,1	0,5—1
	ФЭЧ Н	0,5	0	$8,8 \cdot 10^5$	100	2,5—4
		0	0	$3,6 \cdot 10^5$	40,9	1—2
		0	2	0	0	Нет (j)
TR_1	ФКЭ С	0,5	0	$1,4 \cdot 10^6$	100	1—2
		0	0	$1,1 \cdot 10^6$	78,5	1—2
		0	1	$2,9 \cdot 10^5$	20,7	<0,5
	ФЭЧ Н	0,5	0	$1,2 \cdot 10^6$	100	2,5—4
		0	0	$4,3 \cdot 10^5$	35,8	1—2
		0	1	0	0	Нет (j)
TR_{10}	ФКЭ С	0,5	0	$9,6 \cdot 10^5$	100	1—2
		0	0	$6,4 \cdot 10^5$	66,6	1—2
		0	1	$1,6 \cdot 10^5$	16,6	<0,5
	ФЭЧ Н	0,5	0	$9,4 \cdot 10^5$	100	2,5—4
		0	0	$2,9 \cdot 10^5$	30,8	1—2
		0	1	0	0	Нет (j)

Designations: FKE (C) - culture of chick embryo fibroblasts; FECh (H) - culture of human embryo fibroblasts.

Key: (a) Strain; (b) Cell culture; (c) Concentration (in mg/ml) in covering; (d) DEAE-dextran; (e) dextran sulfate; (f) Titer (in PFU/ml); (g) Effectiveness of seeding (in %); (h) Size of plaques on 5th day (in mm); (i) $L2_{K5}$; (j) None

The effectiveness of seeding on the medium with DEAE-dextran increased by 68-93%; the size of the plaques on the 5th day comprised 0.5-1 mm, and on the 8th day - 2-3 mm. Under a covering which did not contain this polyanion the plaques were smaller: on the 5th day < 0.5 mm, and on the 8th - 1-1.5 mm.

The fpp_1- and fpp_{10}-mutants investigated at 28° did not form plaques on a culture of chick embryo fibroblasts both under a covering which did not contain DEAE-dextran and under a covering with DEAE-dextran in a concentration of 0.5 mg/ml. However, at 37° this polyanion stimulated the formation of plaques by fpp-mutants, and on the 5th day their diameter was 0.5-1.5 mm.

We detected that DS^R-mutants, which are resistant to dextran sulfate at 37°, display a sensitivity to sulphated polysaccharides on a culture of chick embryo fibroblasts at 28°. Under these conditions of cultivation the DS^R_1- and DS^R_3-mutants turned out to phenotypically no different from the initial strain based on the characteristic of sensitivity to sulphated polysaccharides. The addition of DEAE-dextran stimulated an increase in the size and number of plaques, just as in the initial strain. Moreover, at 28° the DS^R-mutants, just as the initial strain, displayed a sensitivity to dextran sulfate, which in concentrations of 2 mg/ml completely suppressed the formation of plaques of the DS^R-mutants.

The thermoresistant (TR) mutants studied did not differ from the initial strain in capacity to form plaques on a culture of human embryo fibroblasts.

It was revealed that during incubation of the initial strain on a culture of human embryo fibroblasts at 37° the sensitivity of the virus to sulphated polysaccharides of agar is manifested more expressedly than on a culture of chick embryo fibroblasts. When DEAE-dextran was added to the agar covering in a concentration of 0.5 mg/ml the effectiveness of seeding was increased 1.8-2.6 times, and the size of the plaques on a culture of human embryo fibroblasts comprised 1-2 mm on the 5th day of incubation at 37° under a covering without DEAE-dextran and 2.5-4 mm in the presence of this substance. Here 80-95% of the plaques had a diameter of 3.5-4 mm (Table 2). The TR- and DS^R-mutants studied did not differ phenotypically from the initial $L2_{K5}$ strain in their capacity to form plaques on a culture of human embryo fibroblasts. In the investigation of the degree of resistance of DS^R-mutants to dextran sulfate complete suppression of plaque formation was revealed on this culture at a concentration of inhibitor of 2 mg/ml, and on a culture of chick embryo fibroblasts under these conditions plaques with a diameter of 0.5-1 mm were detected.

An interesting fact was that the fpp-mutants, which are not able to form plaques on a culture of chick embryo fibroblasts in the absence of DEAE-dextran, turned out to be phenotypically no different from the initial strain in the capacity to form plaques on a culture of human embryo fibroblasts (Fig. 2). The degree of stimulation of plaque formation in fpp-mutants by DEAE-dextran was the same as in the initial strain.

We also detected that the cell culture which is used for reproduction of the virus can influence not only the number and size of plaques, but also the phenotypic manifestation of their morphological peculiarities. Thus in the study of the mt_1- and mt_2-mutants it was revealed that on a culture of human embryo fibroblasts they form "blotched" plaques regardless of the temperature of incubation and the influence of DEAE-dextran in the covering (Table 3). The initial strain under these conditions always formed hyaline plaques (Fig. 3). The size of the plaques formed by the mt-mutants on a culture of human embryo fibroblasts exceeded the size of the plaques formed by the initial strain. As can be seen from Table 3, these differences were manifested most clearly under conditions of cultivation at 28°.

During the investigation of the capacity of mt-mutants to form plaques on a culture of chick embryo fibroblasts it was detected that they did not differ phenotypically from the initial strain and formed hyaline plaques both at 28° and at 37° (Figure 4). During investigation of an infected culture of chick embryo fibroblasts, incubated at 28 and 37°, we did not detect any differences between the initial strain and the mt-mutants either in the effectiveness of seeding, depending on the content of DEAE-dextran in the covering, or in the size of the plaques formed.

Following the staining of a monolayer of human embryo fibroblasts which were infected with mt-mutants and subsequent incubation at 37° after 2 days there was observed a brightening of the "blotched" plaques, which became hyaline. We were interested if this effect was conditioned by the action of the virus on the cell, determining the mottling of the plaque, or is it connected with increased sensitivity of infected cells to neutral red. Upon applying the second layer of agar with neutral red on the 4th, 5th, 6th, and 7th days of incubation at 37° we detected that on the day following administration of the dye "blotched" plaques were always observed. However, in 2-3 days after staining a brightening of the plaques was observed right up to the complete disappearance of mottling.

Discussion

Results of the investigations conducted by us showed that genetic differences of the initial $L2_{K5}$ strain of the herpes virus

Table 3

Influence of conditions of cultivation on the phenotypic manifestation of the characteristic of "blotched plaque" in the mt-mutants of the $L2_{K5}$ strain of the herpes virus

(a) Штамм	(b) Клеточная культура		(c) Температура инкубирования (в градусах)	(d) Концентрация ДЭАЭ-декстрана в покрытии (в мг/мл)	(e) Титр (в БОЕ/мл)	(f) Эффективность посева (в %)	Размер бляшек (в мм) (g)		(j) Морфология бляшек
							(h) 5-е сутки	(i) 8-е сутки	
mt_1	ФЭЧ	Н	37	0,5	$8,3 \cdot 10^5$	100	2,5—4	—	Крапчатые (k)
	ФЭЧ	Н	37	0	$5,1 \cdot 10^5$	61,4	1,5—2,5	—	»
	ФЭЧ	Н	28	0,5	$8,1 \cdot 10^5$	97,5	2—3,5	3—4,5	»
	ФЭЧ	Н	28	0	$1,7 \cdot 10^5$	20,4	1—2	1,5—2,5	»
	ФКЭ	С	37	0,5	$8,2 \cdot 10^5$	100	1—2	—	Прозрачные (l)
	ФКЭ	С	37	0	$6,1 \cdot 10^5$	74,3	1—2	—	»
	ФКЭ	С	28	0,5	$8,4 \cdot 10^5$	102	0,5—1	2—3	»
	ФКЭ	С	28	0	$1,9 \times 10^5$	22,6	<0,5	0,5—1	»
mt_2	ФЭЧ	Н	37	0,5	$1,2 \times 10^6$	100	2,5—4	—	Крапчатые (k)
	ФЭЧ	Н	37	0	$5,7 \times 10^5$	47,5	1,5—2,5	—	»
	ФЭЧ	Н	28	0,5	$9,4 \times 10^5$	78,3	2—3,5	3—4,5	»
	ФЭЧ	Н	28	0	$1,9 \times 10^5$	15,8	1—2	1,5—2,5	»
	ФКЭ	С	37	0,5	$1,4 \times 10^6$	100	1—2	—	Прозрачные (l)
	ФКЭ	С	37	0	$1,1 \times 10^6$	78,5	1—2	—	»
	ФКЭ	С	28	0,5	$1,5 \times 10^6$	107	0,5—1	2—3	»
	ФКЭ	С	28	0	$2,3 \times 10^5$	16,4	<0,5	0,5—1	»

Key: H - human embryo fibroblasts; C - chick embryo fibroblasts; (a) Strain; (b) Cell culture; (c) Temperature of incubation (in degrees); (d) Concentration of DEAE-dextran in covering (in mg/ml); (e) Titer (in PFU/ml); (f) Effectiveness of seeding, in %; (g) Size of plaques (in mm); (h) 5th day; (i) 8th day; (j) Morphology of plaques; (k) Blotched; (l) Hyaline.

and its mutants are manifested phenotypically only under specific conditions of cultivation. Thus the inability of fpp-mutants to form plaques is manifested only during cultivation on cells of chick embryo fibroblasts, but not on cells of human embryo fibroblasts, on which the above stated genetic differences are not reflected on the genotype. In contrast to this type of mutations the mt-mutants are manifested phenotypically only on a culture of human embryo fibroblasts, while a culture of chick fibroblasts is

not a selective medium for their exposure. Thus the phenotypic manifestation of a plaque-forming capability for the herpes virus is determined both by its genotype and also by the genetic organization of the cell in which multiplication of the virus takes place. In this respect our results agree with the findings of other investigators [11, 12], who demonstrated the role of the cell system in manifesting the ability of the herpes virus to form plaques.

At present it is known that such factors as concentration of sodium bicarbonate in the covering, value of pH, and ionic strength of solutions determine the degree of phenotypic expression of the feature of resistance to sulphated polysaccharides [1, 9]. We demonstrated that the type of cell culture and temperature of incubation are significant factors influencing the manifestation of the feature of sensitivity to sulphated polysaccharides by various mutants of the herpes virus. Thus the initial $L2_{K5}$ strain and mutants of the TR, DS^R type display a greater sensitivity to sulphated polysaccharides of agar and dextran sulfate on a culture of human embryo fibroblasts than on a culture of chick embryo fibroblasts. At an incubation temperature of 28° there was also observed a strengthening of the inhibiting action of sulphated polysaccharides. The problem of the mechanism of such a selective manifestation of the investigated features is the subject of a subsequent study.

Just as in the experiments by Thiry [14] with red NDV plaques, we observed a brightening of "blotched" plaques which was conditioned by the action of neutral red on cells infected with the herpes virus. However, in contrast to the red NDV plaques, the formation of which was conditioned by hyperstaining of infected cells, the "blotched" plaques, as we pointed out, are caused by the staining of the symplasts which make up the larger portion of the area of a plaque. The initial strain caused the rapid destruction of infected cells, which conditioned the appearance of bright plaques with sharp edges. The symplasts which formed during infection of a culture of human embryo fibroblasts by mt-mutants [5] were characterized by a relatively low viability and fell off the glass easily. It is possible that under the agar covering in the presence of neutral red the symplasts degenerated rapidly and gave a color to the medium, which caused the clearing up of the plaques.

Conclusions

1. It has been established that the sensitivity of strain $L2_{K5}$ of the herpes virus and its mutants to sulphated polysaccharides of agar depends on the temperature of incubation and is manifested to a greater degree at 28° than at 37°.

2. The capacity of mutants to form plaques and the morphology of the plaques depend on the type of cell culture used for infection; the fpp-mutants turned out to be incapable of forming plaques in the absence of DEAE-dextran only on a culture of chick embryo fibroblasts, but not on a culture of human embryo fibroblasts, while the mt-mutants formed blotched plaques only on the second of these cultures.

3. It was detected that neutral red had an influence on the morphology of plaques which were formed by mt-mutants on a culture of human embryo fibroblasts.

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